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Investigation of the dominance behavior of *Saccharomyces cerevisiae* strains during wine fermentation

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Abstract

During wine fermentation, different strains of *Saccharomyces cerevisiae* compete in the same fermenting must and dominance takes place when one strain overcomes all the others. The purpose of this study was to investigate this phenomenon by identifying *S. cerevisiae* strains endowed with this feature and to test them in laboratory fermentations. First, autochthonous *S. cerevisiae* from Nebbiolo fermentations were isolated, molecularly identified and characterized. Genetically diverse *S. cerevisiae* strains were subsequently subjected to physiological characterization and to micro-scale fermentation, the weight loss kinetics was measured and HPLC analysis was performed at the end of the fermentation. Then, the strains that presented good fermentation characteristics were chosen for further analysis and to determine the dominance feature. For this purpose, couples of strains were co-inoculated in Nebbiolo must and the fermentations were monitored by microbiological and chemical analysis. Two different inoculation approaches were used: co-fermentations in flasks with mixed cells and reactor co-fermentations, in which the cells from the two different strains were kept separate by means of a 0.45 μm filter membrane, which allowed the fermenting must to move freely between the two compartments. During the flask co- fermentations, a minisatellite PCR protocol was applied, in order to differentiate the two strains and determine which one was able to dominate. The protocol included a culture-dependent approach and an independent one. In the first case, DNA extraction was performed on all the colonies scraped off the plates after sampling. In the second case, DNA extraction was performed directly on the fermenting must. The strains that were able to dominate were tested against several *S. cerevisiae* in order to confirm this dominance behavior. Dominance was observed in the early stages of fermentation, as early as 3 days. Combinations of dominant and not-dominant strains were subjected to further tests in a co-fermentation reactor system, in order to perform single-strain analysis so as to obtain a better understanding of the dominance behavior. Surprisingly, the results obtained in the flask co-fermentations were not confirmed. In fact, the two strains, one which was hypothesized to be dominant and the other not-dominant, coexisted throughout the fermentation period. The results of

this study suggest that the dominant behavior of *S. cerevisiae* is only expressed when they sense other yeasts in the same environment.

Keywords: Wine fermentation; *Saccharomyces cerevisiae;* cell-to-cell contact; competition between strains; dominance.

1. Introduction

Natural microbial ecosystems are characterized by a high level of biodiversity. In such a community, microorganisms compete for space and resources. Research efforts that have focused on understanding the mechanisms responsible for the coexistence or dominance of these microorganisms have highlighted the multitude of strategies that they can employ when found in the same ecosystem. From the information collected in studies in this specific research area, it is becoming evident that microorganisms of the same species are able to interact and that they may develop competitive or cooperative behavior. Through a study of such phenomena, it is possible to understand how specific individuals, within a population consisting of representatives of the same microbial species, may dominate or decline (Hibbing et al., 2010).

The fermentation of grape juice into wine is a complex microbiological process that involves interactions between yeasts, bacteria and filamentous fungi. Naturally, during fermentation, many different species and strains undergo sequential substitution. This substitution may be explained by the competitive exclusion of those microorganisms that are less competitive (Arroyo-Lopez et al., 2011). The substitution of species normally takes place because of the changes that occur in the must matrix, which is becoming wine. Yeasts, such as *Kloeckera/Hanseniaspora, Torulaspora, Candida* and *Zygosaccharomyces*, are commonly present on the surface of grapes. After crushing, the grape must becomes a selective ecosystem because of the high concentration of sugars and nitrogen, salts, trace elements and oxygen amount. Poorest varieties in nitrogen, salt and trace elements limit the yield in biomass of the cells (Monteiro and Bisson, 1991), while a low oxygen

content promotes *S. cerevisiae* as it is more able to produce cellular fatty acids (Mauricio et al., 1998) Finally temperature, increasing naturally because of the *S. cerevisiae* fermentative activity represents an inhibition factor for others species (Goddard, 2008). Certain species, such as *Kloeckera* and *Hanseniaspora,* grow more during the first hours after the crushing, due to the higher counts on the grape skins and can start fermenting during the first 1-3 days of the fermentation (Pèrez-Nevado et al., 2006). If no starter culture is used, *Saccharomyces cerevisiae* contamination normally occurs from the winery environment. The winery environment can be a source of yeast inoculation, and this yeast may be preserved for years.

Although the predominance mechanism of *S. cerevisiae* on others genera is quite known (Fleet and Heard, 1993; Nissen et al., 2003; Salvadò et al., 2011), few studies on the competition between species of the same genera are present in literature (Arroyo-Lopez et al., 2011), and little is known about competition between strains of the same species, with the exception of studies on killer factors (Barandica et al., 1999; Jacobs and van Vuuren, 1991; Psani and Kotzekidou, 2006).

This study has focused on: (i) the identification and characterization of potential autochthonous dominant *S. cerevisiae* from Nebbiolo musts from the Piedmont region, Northwest Italy; (ii) the investigation of *S. cerevisiae* intraspecies competition during wine fermentations in which the cells of the different strains were mixed or kept separated. The final aim of this study was to investigate the behavior of *S. cerevisiae,* when competing in the same fermenting must, in order to obtain new insights into the competition phenomenon.

2. Materials and methods

2.1. Yeast strains and growth media

Ninety- nine strains of indigenous *S. cerevisiae,* isolated from the spontaneous fermentation of Nebbiolo must, and the commercial *S. cerevisiae* BRL97 (Lallemand, Montreal, Canada), have

been examined in this study. The yeasts were maintained at 4°C on WLN (Wallerstein Laboratory Nutrient agar) medium (Oxoid, Milan, Italy).

2.2. Molecular identification and characterization of the isolates

After DNA extraction, as described by Cocolin et al. (2000), the *S. cerevisiae* were identified using a species-specific PCR, according to Manzano et al. (2005), and then characterized through a Sau-PCR protocol, as suggested by Cocolin et al., (2006). The profiles were digitally transformed and processed into clusters using the Bionumerics statistic software (Applied Maths, Sint-Martens-Latem, Belgium).

2.3. Physiological characterization

Apart from the molecular characterization, a physiological characterization was also performed to test the hydrogen sulfide production and resistance to sulfur dioxide and ethanol. The Biggy agar medium (Oxoid) was used for the hydrogen sulfide production and its production was evaluated on the basis of the intensity of the brown color after growth. Sulfur dioxide and ethanol resistance were tested in Yeast Nitrogen Base without amino acids (Sigma, Milan, Italy) with glucose 2% (w/v). Sulfur dioxide resistance was tested at 50 – 100 – 150 – 200 mg/l of sulfur dioxide, which were obtained by adding different concentrations of potassium metabisulfite to the YNB with a pH of 4. Ethanol resistance was tested at concentrations of 14 – 16 – 18 – 20% (v/v). Growth was evaluated after 3 days of incubation at 30°C.

2.4. Micro-fermentation to test the technological performances of the strains

On the basis of the physiological and Sau-PCR characterization, 24 strains of *S. cerevisiae* were selected and tested in micro-fermentation tests. *S. cerevisiae* BRL97 was used as the control. In order to prepare the cultures for inoculation, Nebbiolo grape must (reducing sugars 270 g/L, assimilable yeast nitrogen content 200 mg/L) was used. One colony was inoculated in 10 ml of pasteurized must (100°C for 10 min) and incubated at 25°C. After 72 hours, 30 ml of pasteurized

must was added to the culture and incubated at the same temperature for an additional 72 hours. Finally, 360 ml of pasteurized must was added to the active inoculum in a bubbler capped bottle filled with paraffin oil, as described by Romano et al. (2003). The fermentations were run at 25°C for 15 days and were monitored daily to measure the weight loss. At the end of the fermentation, the residual glucose and fructose, the glycerol and ethanol production and the malic acid content were quantified by means of HPLC (Thermo Electron Corporation, Waltham, MA, USA) equipped with a UV detector (UV100), set to 210 nm, and a refractive index detector (Waters 2414, Waters Corporation, Milford, MA, US). The analyses were performed isocratically at 0.8 mL/min and 65 °C with a 300x7.8 mm i.d. Aminex HPX-87H cation exchange and a Cation H+ Microguard cartridge (Bio-Rad Laboratories, Hercules, CA, USA), using 0.0026N H_2SO_4 as the mobile phase (Giordano et al., 2009; Schneider et al., 1987). Statistical analysis was performed using the statistical software package IBM SPSS Statistics (version 19.0; IBM Corp., Armonk, NY, USA). Principal component analysis (PCA) was used to evaluate the performance and carry out the selection of the yeast strains. The components with eigenvalue greater than 1 were extracted, no rotation was used.

2.5. Minisatellite profiling

Fourteen strains showing good technological performances, determined from the micro-fermentations, were selected and characterized through the amplification of minisatellite *SED1* (Marinangeli et al., 2004).

2.6. Trial fermentations to study the dominance phenomenon

Two fermentation set-ups were used to study *S. cerevisiae* intraspecies competition. First, strains showing different minisatellite profiles were divided into 48 couples and inoculated in flasks with loose screw cap with a final concentration of 10^5 cell/ml per strain, in 100 ml of pasteurized must, in order to check the dominance behavior. The fermentations were incubated at 25°C for 10 days. Subsequently, in order to obtain better insight into the dominance behavior, two couples of strains, one behaving in a dominant manner and the other in a non-dominant way, were tested in mixed fermentations in a co-fermentation bioreactor, and were sampled at five and ten days. Single strain

fermentations were also performed as controls. The bioreactor co–fermentation system was designed as previously described by Di Cagno et al. (2010). It was composed of two glass compartments with loose screw cap, separated by a filter membrane with a porosity of 0.45 µm (Fig. 1). A plexiglass system was designed to apply the necessary pressure to the glass system. Each bioreactor sector was filled with 100 ml pasteurized must and inoculated with the two strains to be tested (10^5 cell/ml). Chemical homogeneity in the alcohol, sugars and acids was tested between the two compartments by means of HPLC, as described above. All the experiments were conducted in duplicate.

2.7. Verification of dominance

The capability of one strain to dominate over another was verified during mixed fermentation through DNA approaches. Three different strategies were adopted: a) colony-by-colony analysis: mixed fermentations were sampled daily on the WLN. Dominance was checked by picking 10 colonies from the WL plates, and these were subjected to DNA extraction, as described by Cocolin et al. (2000), and subjected to the *SED1* PCR protocol; b) extraction of bulk colonies: 1 ml of Ringer's solution (Oxoid) was added to plates containing 30 to 300 colonies and were scraped off the plate using a sterile spreader. DNA extraction was conducted on 300 µl of the homogenate, as described above. The DNA, standardized at 100 ng/µl, was subjected to the *SED1* PCR protocol; c) direct extraction of DNA from the fermenting must: 1 ml of fermenting must was subjected to DNA extraction using the DNeasy plant mini kit (Qiagen, Milan, Italy), according to the manufacturer's instructions. The DNA, standardized at 100 ng/µl, was subjected to the *SED1* PCR protocol.

3. Results and discussion

The literature is rich in studies that describe the mechanisms through which *S. cerevisiae* dominates wine fermentation. (Fuqua et al., 1996, Goddard 2008, Hayashi et al., 1998, Kleerebezem et al., 1997, Piskur et al., 2006, Reguera 2011, Richard et al., 1996) *S. cerevisiae* yeasts are very

competitive, due to a combination of properties, such as fast growth, efficient glucose consumption, good ability to produce ethanol and a higher tolerance to environmental stresses (Piskur et al., 2006). Goddard (2008) has recently suggested that *S. cerevisiae* eventually dominates wine fermentations by modifying the environmental temperature through heat production during vigorous fermentations. Unfortunately, neither of these hypotheses can explain competition within the same genera or species. Quorum sensing has been described for bacteria (Fuqua et al., 1996, Kleerebezem et al., 1997) and for yeast (Reguera 2011), in particular for the coordination of the metabolism in *S. cerevisiae* at high cell densities, with acetaldehyde as the probable signal molecule (Richard et al., 1996), and for the stimulation of meiosis and sporulation with bicarbonate as signal molecule (Hayashi et al., 1998). However, such mechanism can not explain the change in behavior when cells are in communication for metabolites, but not in contact.

3.1. Physiological and genetic characterization

A collection of 99 isolates obtained from spontaneous fermentation of Nebbiolo musts were subjected to molecular identification and characterization. All the isolates were positively amplified using a specific set of primers for *S. cerevisiae,* as described by Manzano et al. (2005). Electrophoretic profiles of Sau-PCR were analyzed with Bionumerics software and a total of 15 clusters were obtained (data not shown). A physiological characterization of the isolates showed that 98% of them resisted ethanol and sulfur dioxide stress, but some were able to grow faster than others (data not shown). Most of the collection produced high or medium levels of hydrogen sulfide, and only two strains displayed a very low production. On the basis of the molecular and physiological characterizations, 24 different strains were selected and tested for their fermentative performances, in micro-fermentation tests, together with the control strain BRL97.

3.2. Microfermentation tests

The fermentations were followed by measuring the weight loss, which is directly correlated to sugar consumption. The strains showed similar fermentative trends, with a peak of CO_2 production of between 0.2 and 0.35 g/h at 50 hours of fermentation (data not shown). The chemical composition of the musts at the end of the fermentation trials are shown in Table 1. All the fermentations, except for 7 strains, ended up with a residual sugar content of less than 10.0 g/L, with good ethanol contents (above 14.0 % vol.), and adequate produced glycerol contents (between 7.0 and 9.0 g/L). All the fermentations showed a lower acetic acid content than 0.5 g/L, except for strain #2, which produced a slightly higher content (0.515 g/L). BRL97, which was used as a reference, showed a better performance than most of the tested strains, with a limited acetic acid production (0.386 g/L) and a good fermentation yield (0.450 g ethanol/g sugar). On the basis of the chemical composition results, a principal component analysis was conducted to evaluate the performances of the strains. The first two components were extracted with PC1 (56.1 % of explained variance) and were mainly correlated to the sugar and ethanol contents, while PC2 (17.3 % of variance) was correlated to the fermentation yield and slightly correlated to the acetic acid production, which does not seem to be directly related to the sugar consumption or ethanol production. The two-dimensional plots of the variables (a) and the fermentation trials (b) are shown in Figure 2 for the two first principal components: the fermentations which showed the best results are positively correlated to PC1, especially in the middle and lower part of the plot (while PC2 is negatively correlated to the ethanol yield). Fourteen strains showing good fermentation performances were selected for the subsequent mixed fermentations tests. These strains were mainly located in the middle and on the right part of the plot. Outliers and those strains that left high-residual sugars after fermentation were excluded. These strains were then subjected to characterization of the *SED1* region, encoding for the most abundant cell wall glycoprotein of the *S. cerevisiae* stationary phase-cells (Mannazzu et al., 2002).

3.3. Dominance results

For the *S. cerevisiae* strains employed in this study, a dimorphism was observed in the molecular weight of the *SED1*-PCR amplification product. Some strains showed a band with 1 Kbp molecular weight, while the remaining ones had a PCR product of about 1.2 Kbp (Fig. 3). This difference in the molecular weight of the *SED1* amplification product could discriminate the strains in the mixed culture, and thereby permitted the dominant behavior to be checked quickly,. On the basis of this evidence, 48 couples of *S. cerevisiae* were inoculated in mixed fermentations in order to determine which strains were able to dominate. The overall results of the mixed fermentations, in terms of dominance, are presented in Table 2. After the minisatellite analysis of the DNA extracted from the pooled colonies on the plates and directly from the must, a dominance of one strain was revealed in 35.5% of the cases, while co-dominance (permanence of both bands) was observed in the remaining trials. One band was more intense than the other in 12.5% of the combinations suggesting partial dominance. The *SED1* profiles of the representative fermentation trials are presented in Figure 4. In every case of dominance one strain became dominant at the very beginning of the fermentation, within the first 2-3 days. The minisatellite profiles did not change from this point onwards, thus underlining that the dominant strain remained stable until the end of the fermentation (Fig. 5).

The competition between *S. cerevisiae* and the outcompeted non-*Saccharomyces* yeast during natural and simulated fermentations has been studied extensively (Holm Hansen et al., 2001; Mendoza et al., 2007; Nissen and Arneborg, 2003). The early dominance observed in this study is in agreement with the results of Nissen at al. (2003), who demonstrated that the early death on non-*Saccharomyces* strains starts at 2 days of fermentation, even in the case of competition between different species, namely *S. cerevisiae* against *Lachancea (Kluyveromyces) thermotolerans* and *Torulaspora delbrueckii*. It is possible to speculate that nutrient limitation due to must composition, temperature and the presence of growth inhibitory compounds could affect the imposition of most adaptable species. This hypothesis can be applied also when considering competition between strains of the same species. In Nissen et al. (2003), it was speculated that the growth arrest of non-*Saccharomyces* cells was due to a cell-to-cell contact mechanism that depended on the presence of

viable cells of *S. cerevisiae.* Apparently, the ability of *S. cerevisiae* to arrest the growth of wine yeasts in mixed cultures was not restricted to a single strain, but was instead a common feature of this species. However, the results of the present study have shown that strains behave differently. According to this hypothesis, the arrest of the growth of non-*Saccharomyces* cannot only be correlated to the Crabtree effect (Thomson et al., 2005), to a lower tolerance of ethanol (Fleet and Heard, 1993) or to the concept of a fitness advantage (Salvadò et al., 2011). Ecological studies have indicated that killer activity, through the production of a toxic compound by one strain that excludes the others from its habitat, could be a competitive or "self-protection" mechanism (Starmer et al., 1987; Zagorc et al., 2001). Killer activity has been observed in numerous genera of wine yeasts, such as *Saccharomyces, Candida, Cryptococcus, Debaryomyces, Hanseniaspora, Kluyveromyces, Pichia, Williopsis* and *Zygosaccharomyces* (Zagorc et al., 2001). Several papers have been published about killer toxins (Flagelova et al., 2003; Yap et al., 2000). No killer activity, investigated according to Zagorc et al. (2001) has been detected in the present study (data not shown).

Another case of competition due to a cell-to-cell contact was observed by Arroyo-Lopez et al. (2011), who investigated the interaction between two different species of *Saccharomyces*, namely *S. cerevisiae* and *S. kudriavzevii*. In co-culture with *S. kudriavzevii, S. cerevisiae* was the most competitive yeast, whereas, at low temperatures, *S. kudriavzevii* grew faster than *S. cerevisiae* in the early stages of fermentation. However, *S. kudriavzevii* always arrested its growth earlier than *S. cerevisiae*, at any temperature assayed.

Little information is currently available regarding what happens when two strains of *S. cerevisiae* are in competition. Cheraiti et al. (2005) described differences in redox potential between single-strain fermentations and mixed-strain fermentations of *S. cerevisiae.* Howell et al. (2005) studied differences in aroma profile of wine produced with mixed cultures of *S. cerevisiae.*

On the basis of the results presented in Table 2, four strains were selected for further studies in bioreactor co-fermentations. Combinations of strains 1 (dominant) and 14 (not dominant) as well as

12 (dominant) and 11 (partially dominant: in combination with some *S. cerevisiae,* strain 11 dominated but in other cases it did not) were inoculated in the bioreactor. Although in mixed fermentations, the strains showed a decrease in the number of cells for non-competing strains (i.e. 14 and 11) as they were not detectable by *SED1*-PCR, this behavior was not observed in the bioreactor co-fermentations. We assumed that the non dominant strain was at the count below 10^5 cfu/ml. This limit is deduced considering the dilution of the plate from which DNA extraction was performed (data not shown). The dominance phenomenon disappeared totally for both of the tested couples, as established from the counts on the WLN plates (Tab. 3). Values show that both the strains reach the same cell density in the case of the bioreactor. It can be speculated that, in the conditions found in the bioreactor, where the strains do not sense each other, dominance does not take place. Metabolite exchange between the two compartments was verified by means of HPLC in order to exclude their non-homogeneous distribution and the results pertaining to the alcohol, fructose, glucose, glycerol, acetic acid, citric acid, tartaric acid and malic acid suggested that both sectors were equivalent. Data of sugars and alcohol concentration are not significantly different. Highest difference between the two compartments was detected for sugars (3%) (data not shown), however this value is lower than the instrumental reproducibility. The results obtained from the bioreactor fermentation again compared well with those obtained by Nissen et al. (2003), who used a dialysis tube to separate different yeast species. In that case, non-*Saccharomyces* yeasts were inoculated outside the dialysis tube, while *S. cerevisiae* was grown inside the dialysis tube. When compartmentalized, the non-*Saccharomyces* yeasts did not decrease. The evidences presented here could be explained by the mechanism proposed by Nissen et al. (2003), who postulated that growth arrest is due to cell-to-cell contact or microenvironment contact. In these cases, cells compete for space when in high densities and in cell-to-cell contact.

In conclusion, in 35.5% of the combinations presented in this study, one of the two strains of *S. cerevisiae* died off or decreased in cellular density so much that it was undetectable in the mixed fermentations. On the contrary, competition did not take place when the same strains were

separated and did not compete for space, even when they shared the same fermenting must. These results can be considered a contribution to the better understanding of the competition mechanism that occurs between strains of *S. cerevisiae* and suggest the necessity of extending the investigation to strain-to-strain interactions.

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Table 1. Average residual reducing sugars, malic acid, ethanol, glycerol and acetic acid composition from the laboratory fermentation trials (n = 2) of the selected 24 *S. cerevisiae* isolated from Nebbiolo fermentations. BRL97 (Lallemand) was used as a control.

Strain	**Reducing sugars g/L**			**Glucose/fructose ratio**			**Ethanol % vol.**			**Glycerol g/L**			**Acetic acid g/L**			**Malic acid g/L**			**Yield g ethanol/g sugar**		
1	5,89	±	1,33	0,368	±	0,087	14,85	±	0,25	7,88	±	0,08	0,495	±	0,046	1,62	±	0,12	0,442	±	0,010
2	42,36	±	6,52	0,189	±	0,035	12,69	±	0,09	6,65	±	0,07	0,515	±	0,028	1,62	±	0,12	0,439	±	0,016
3	11,35	±	2,74	0,237	±	0,029	14,81	±	0,09	7,32	±	0,10	0,455	±	0,011	1,74	±	0,04	0,451	±	0,008
4	21,60	±	2,86	0,104	±	0,003	13,85	±	0,05	6,82	±	0,15	0,490	±	0,002	1,77	±	0,06	0,439	±	0,006
5	10,28	±	0,35	0,190	±	0,003	14,80	±	0,56	6,77	±	0,06	0,454	±	0,003	1,64	±	0,06	0,448	±	0,017
6	7,87	±	5,06	0,363	±	0,282	14,83	±	0,17	7,65	±	0,45	0,475	±	0,045	1,61	±	0,03	0,445	±	0,004
7	5,54	±	3,16	0,605	±	0,464	15,02	±	0,06	8,57	±	0,60	0,464	±	0,048	1,72	±	0,07	0,447	±	0,004
8	13,10	±	1,58	0,187	±	0,010	14,54	±	0,19	7,60	±	0,45	0,435	±	0,033	2,06	±	0,08	0,445	±	0,003
9	6,07	±	2,37	0,407	±	0,227	14,80	±	0,03	7,91	±	0,62	0,468	±	0,035	1,65	±	0,07	0,441	±	0,003
10	8,13	±	4,57	0,421	±	0,300	14,78	±	0,01	7,65	±	0,45	0,414	±	0,002	2,04	±	0,04	0,444	±	0,007
11	8,32	±	4,22	0,406	±	0,280	14,52	±	0,56	7,64	±	0,58	0,404	±	0,019	2,13	±	0,17	0,436	±	0,010
12	4,59	±	2,05	0,520	±	0,284	14,79	±	0,30	7,19	±	0,07	0,459	±	0,009	1,45	±	0,08	0,439	±	0,012
13	3,70	±	0,50	0,748	±	0,152	14,79	±	0,11	8,08	±	0,20	0,462	±	0,014	1,66	±	0,00	0,437	±	0,004
14	9,47	±	0,18	0,253	±	0,001	14,22	±	0,19	7,33	±	0,09	0,425	±	0,011	1,99	±	0,02	0,429	±	0,006
15	4,66	±	0,84	0,538	±	0,142	14,69	±	0,03	7,80	±	0,16	0,436	±	0,011	1,70	±	0,02	0,436	±	0,002
16	6,30	±	0,55	0,450	±	0,029	15,19	±	0,44	8,24	±	0,34	0,463	±	0,001	2,10	±	0,07	0,453	±	0,014
17	4,81	±	0,16	0,717	±	0,037	14,88	±	0,37	8,32	±	0,22	0,431	±	0,051	2,20	±	0,04	0,441	±	0,011
18	6,61	±	3,22	0,558	±	0,371	14,83	±	0,36	8,07	±	0,14	0,428	±	0,042	2,17	±	0,03	0,443	±	0,016
19	3,65	±	0,25	0,938	±	0,111	15,06	±	0,06	8,79	±	0,22	0,343	±	0,017	1,92	±	0,02	0,445	±	0,002
20	5,78	±	3,09	0,529	±	0,360	14,92	±	0,18	7,15	±	0,02	0,364	±	0,021	1,77	±	0,06	0,444	±	0,000
21	17,94	±	5,56	0,145	±	0,016	14,06	±	0,17	6,78	±	0,35	0,460	±	0,020	1,89	±	0,05	0,439	±	0,004
22	4,21	±	0,87	0,625	±	0,211	15,27	±	0,00	8,12	±	0,15	0,477	±	0,030	1,65	±	0,02	0,452	±	0,002
23	6,22	±	1,33	0,396	±	0,129	15,12	±	0,28	7,39	±	0,15	0,356	±	0,002	1,80	±	0,05	0,451	±	0,006
24	30,33	±	2,52	0,129	±	0,013	13,42	±	0,50	6,51	±	0,52	0,349	±	0,000	1,75	±	0,09	0,440	±	0,012
BRL97	6,04	±	0,46	0,401	±	0,039	15,09	±	0,57	7,06	±	0,34	0,386	±	0,001	1,70	±	0,05	0,450	±	0,016

Table 2. Schematic representation of the results obtained from the competition experiments performed on mixed fermentations. *S. cerevisiae* strains with different minisatellite *SED1* profiles (in black, strains with the 1.2 kb PCR product, in white the ones with the 1 kb PCR product) were inoculated in Nebbiolo must. The data presented in this table were obtained from direct DNA extraction from must and from colonies pooled from plates. Dominance during the fermentation process is shown using the black and white code. In the case of co-dominance, a gray color is used. Strains 1 and 12 dominated in most of the cases, while strains 14, 17 and 18 never dominated. *asterisks indicate cases of partial dominance.

	1	3	6	7	9	12	13	22
10	1/10	10	6/10	10	10	12	13/10	22/10
11	1	3	11*	11*	9/11	12	13/11	11*
14	1	3	6/14	7/14	9/14	12	13/14	22
16	1/16	3/16	6/16	7/16	16*	12/16	13/16	22/16
17	1	3*	6	7/17	9/17	12	13/17	22
18	1	3*	6/18	7/18	9/18	12	13/18	22/18

Table 3. Counts on WLN (log colony forming units/ml ± SD) from the couples chosen for the bioreactor test. Counts values are associated to SD value. Dominant strains are numbers 1 and 12. Non dominant strains are numbers 11 and 14. Mixed fermentations were performed in Nebbiolo must in all the tests. Values for pure culture fermentations and mixed cultures fermentations in the reactor are shown. In the last column, cumulative counts for both the strains present in mixed culture fermentations (flask) are shown.

		5 days					
S. cerevisiae strains		Pure cultures		Mixed cultures in the reactor		Mixed cultures in flasks	
11+12	11	8.21	± 0.12	8.17	± 0.23	8,16	± 0,00
	12	8.28	± 0.13	8.04	± 0.47		
1+14	1	8.14	± 0.25	7.92	± 0.32	8,07	±0,02
	14	8.05	± 0.00	8.01	± 0.17		

		10 days					
S. cerevisiae strains		Pure cultures		Mixed cultures in the reactor		Mixed cultures in flasks	
11+12	11	8.25	± 0,04	8.38	± 0.00	8.16	± 0.05
	12	8.27	± 0,11	8.07	± 0.00		
1+14	1	8.14	±0,04	8.03	± 0.01	8.07	± 0.01
	14	8.10	± 0,09	7.84	± 0.24		

Figure legends

Figure 1. Co-culture bioreactor composed by two compartments. The filter membrane allows the exchange of metabolites keeping separated cells belonging to two different strains, inoculated separately at the same cell density in the two compartments.

Figure 2. Projection of each chemical variable of the fermentation (a) and of the yeast strains (b) in the plane defined by the two first principal components.

Figure 3. Dimorphism of the *SED1* region of the 14 selected strains of *S. cerevisiae.* Lane 1, molecular weight marker 1kb (Sigma); lanes 2, 3, 4, 5, 6, 9, 10 and 15, strains of *S. cerevisiae* giving a 1 kb *SED1* minisatellite amplification, lanes 7, 8, 11, 12, 13 and 15, strains of *S. cerevisiae* giving a 1.2 kb *SED1* minisatellite amplification; lane 16, negative control.

Figure 4. Results of the *SED1* amplification during mixed fermentation performed in Nebbiolo must as described in the text (2.4). Lane 1, molecular weight marker 1 kb (Sigma); lane 2, positive control; lane 3 and 4, couple 1/16 showing co-dominance; lanes 5 and 6, couple 3/16 showing co-dominance; lanes 7 and 8 couple 9/16 showing partial dominance; lanes 9 and 10 couple 1/17 showing dominance of strain 1; lanes 11 and 12 couple 7/16 showing co-dominance; lanes 3, 5, 7, 9 and 11, minisatellite profiles of *S. cerevisiae* DNA directly extracted from must after 5 days of fermentation; lanes 4, 6, 8, 10 and 12 minisatellite profiles of *S. cerevisiae* DNA extracted from the total colonies scraped off the plate after 5 days of fermentation sampling. In one case (lane 7 and 8) it is possible to recognize a phenomenon of partial dominance. For DNA extracted directly from must, one band is more visible than the other, probably due to a higher concentration of one strain, difference that is not possible to observe after the growth (pooled colonies from plate).

Figure 5. *SED1* profiles for individual colonies randomly isolated during the flask mixed fermentation of *S. cerevisiae* strain 14, which gives a 1 kb PCR product, and *S. cerevisiae* strain 1, which gives a 1.2 kb molecular weight band. Figure includes several isolates from different sampling points and represents an example of dominance at three days of fermentation. Lanes 1 and 21, molecular marker 1kb (Sigma); lane 2, *S. cerevisiae* strain 14; lane 3, *S. cerevisiae* strain 1;

lanes 4-6, isolates from day 0 of fermentation; lane 7, negative control; lanes 8-12, isolates at day 1 of fermentation; lanes 13-17, isolates at day 2 of fermentation; lanes 18-20, 22,23, isolates at day 3 of fermentation; lanes 24-28, isolates at day 4 of fermentation.

Figure 1

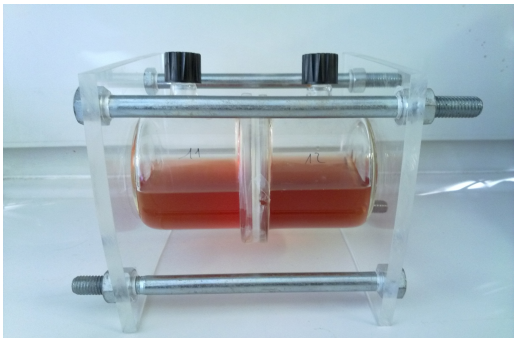

Figure 2

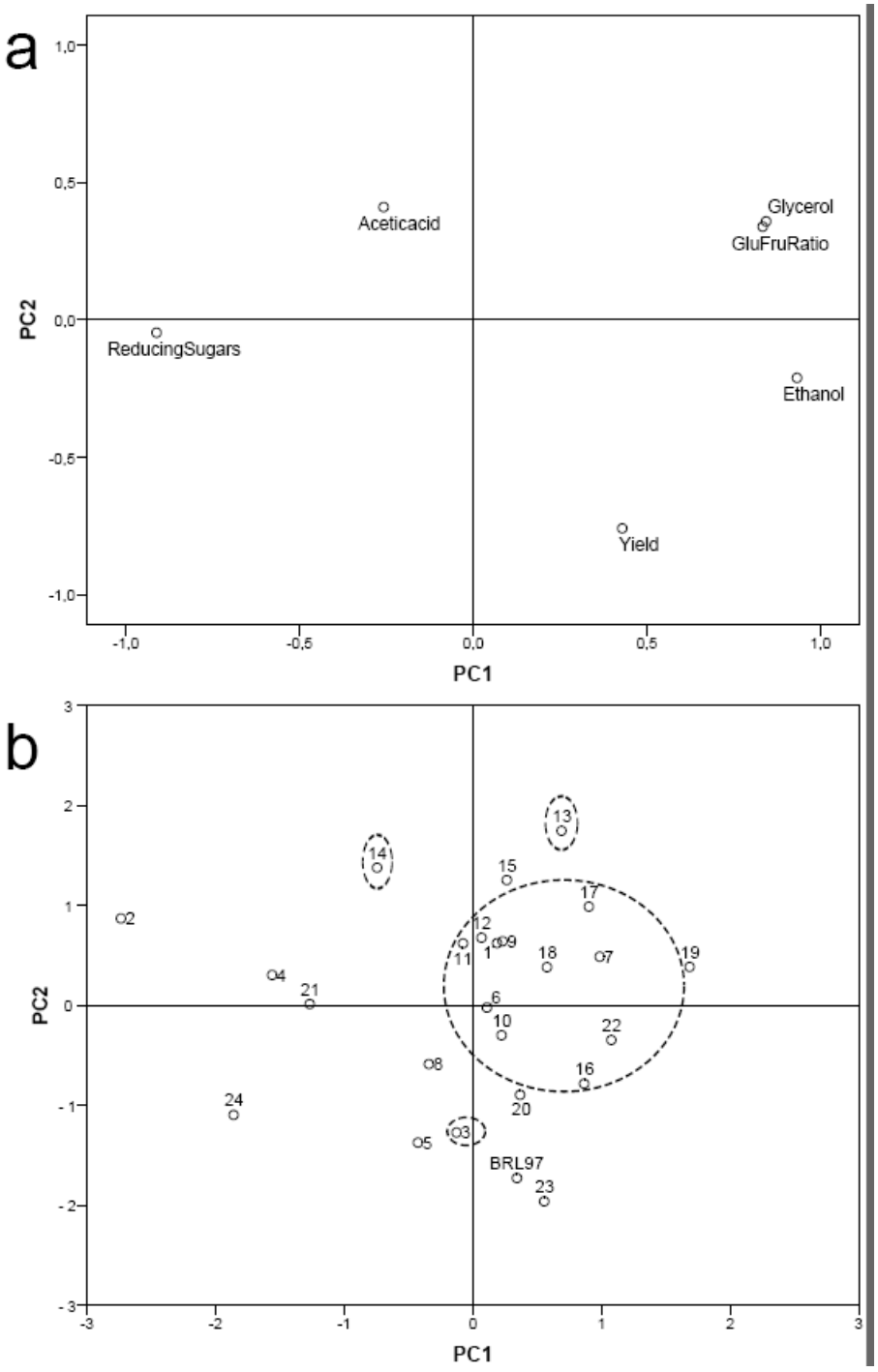
a
Aceticacid
Glycerol
GluFruRatio
ReducingSugars
Ethanol
Yield
PC1
PC2
b
BRL97
PC1
PC2

Figure 3

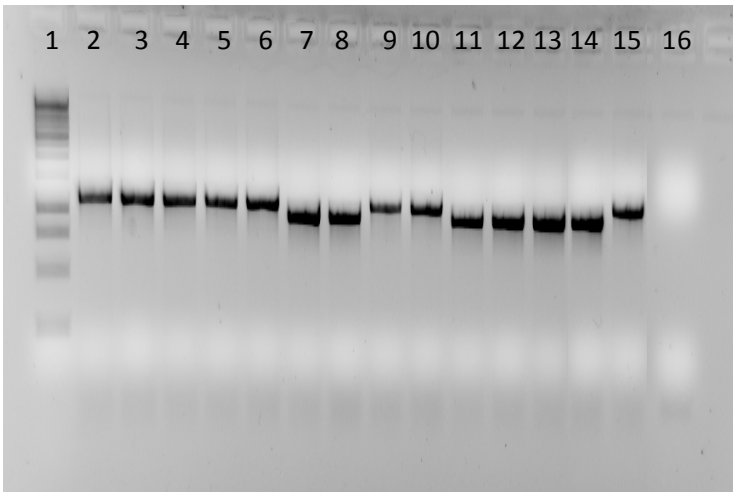

Figura 4

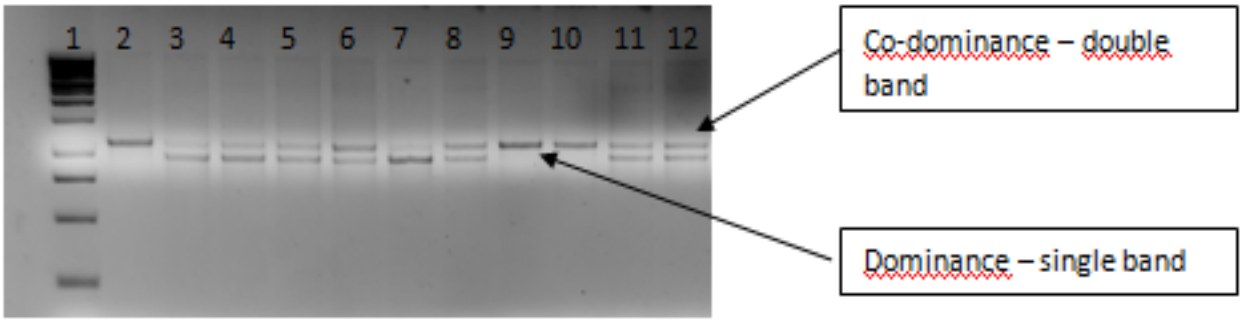
1 2 3 4 5 6 7 8 9 10 11 12
Co-dominance – double band
Dominance – single band

Figura 5